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In utero exposure to cigarette smoke and effects across generations: a conference of animals on asthma

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ABSTRACT

Background: The prevalence of asthma and chronic obstructive pulmonary disease (COPD) has risen markedly over the last decades and is reaching epidemic proportions. However, underlying molecular mechanisms are not fully understood, hampering the urgently needed development of approaches to prevent these diseases. It is well established from epidemiological studies that prenatal exposure to cigarette smoke is one of the main risk factors for aberrant lung function development or reduced fetal growth, but also for the development of asthma and possibly COPD later in life. Of note, recent evidence suggests that the disease risk can be transferred across generations, i.e. from grandparents to their grandchildren. While initial studies in mouse models on *in utero* smoke exposure have provided important mechanistic insights, there are still knowledge gaps that need to be filled.

Objective: Thus, in this review we summarize current knowledge on this topic derived from mouse models, while also introducing two other relevant animal models: the fruit fly *Drosophila melanogaster* and the zebrafish *Danio rerio*.

Methods: This review is based on an intensive review of Pubmed-listed transgenerational animal studies from 1902-2018 and focuses in detail on selected literature due to space limitations.

Results: This review gives a comprehensive overview of mechanistic insights obtained in studies with the three species, while highlighting the remaining knowledge gaps. We will further discuss potential (dis)advantages of all three animal models.

Conclusion/Clinical relevance: Many studies have already addressed transgenerational inheritance of disease risk in mouse, zebrafish or fly models. We here propose a novel strategy for how these three model organisms can be synergistically combined to achieve a more detailed understanding of *in utero* cigarette smoke induced transgenerational inheritance of disease risk.

INTRODUCTION

Prenatal exposure to cigarette smoke is a recognized risk factor for reduced fetal growth and impaired lung function development (reviewed in [1,2]). Prenatally exposed individuals therefore are at greater risk to develop asthma, both in childhood [3,4] to adolescence [5] and adulthood [6–8], and possibly also chronic obstructive pulmonary disease (COPD) [9] with ageing [10–12]. Despite decreasing smoking rates in several countries, numbers of women who smoke during pregnancy have remained unacceptably high [13,14]. In addition, pregnant women are starting to use e-cigarettes at rapidly increasing rates almost equal to conventional cigarettes [15]. These are suspected to affect the fetal health similar to conventional cigarettes [16].

Furthermore, it has been shown that maternal smoking during pregnancy appears to increase her grandchild's risk for lower birth weight (boys only) [17] and for asthma, independent of the maternal smoking status [18–20]. Moreover, prenatal exposure of the father via his mother appears to affect the risk for persistent wheeze in his daughter [21].

So far, studies have exclusively investigated the maternal line for priming of respiratory disease via smoking. However, a very recent multicenter study in Northern Europe demonstrated that also paternal smoking might increase the risk for asthma in offspring [22]. The risk was markedly higher when fathers smoked during puberty, and this was independent from whether he quit smoking years before the child was born or how much the father smoked. A possible explanation for this could be that during puberty, primordial germ cells start to develop into spermatogonia in the testes from which mature spermatozoa arise. This developmental window could therefore be particularly susceptible to environmental insults [22].

If these observations hold true, the consequences of parental or grandparental smoking will last for decades. Beyond intensifying anti-tobacco campaigns, it is therefore essential to create preventative strategies to ensure normal lung development in exposed offspring requiring a clear understanding of the mechanisms how smoking affects fetal lung development. To accomplish this task, animal models are an important prerequisite.

The present review summarizes current knowledge of how prenatal cigarette exposure influences fetal development and molecular pathways in mice (*Mus musculus*). We will further discuss the usefulness of more simple organisms such as the fruit fly (*Drosophila melanogaster)*, and the zebrafish (*Danio rerio*) to advance our understanding on early life origins of chronic lung diseases. Finally, we propose an integrative approach for using these species for transgenerational research.

Murine models of prenatal smoking

Mice reach sexual maturity at 6-8 weeks of age and have a high reproduction rate yielding 5-8 pups per litter. Their gestation time lasts usually 21 days (**Fig. 1A**) with lung development starting at embryonic day (ED) 8 by formation of the lung bud. The timing of the following pseudoglanduar (ED 9.5-16.6), canalicular (ED 16.6-17.4), saccular (ED 17.5-postnatal day (PND) 5) and alveolar stages (PND 5-30) is clearly defined [23]; thus allowing to investigate the influence of maternal smoking during specific developmental windows (**Fig. 1A**). In addition, the development, morphology and cellular composition of the human (**Fig. 2A and 2B**) and murine lung (**Fig. 2C and 2D**) and the immune system have some close similarities, facilitating the translation of findings to the human organism as compared to other species. The large variety of available knock-out mice further helps to investigate the contribution of distinct molecular pathways to disease development. Until now, a number of murine models of prenatal smoking have been developed (e.g.[24–26]). One important criterion for the quality of these models is whether or not they are able to recapitulate the clinical hallmarks seen in prenatally smoke exposed children.

Models of active cigarette smoking during pregnancy

Intrauterine growth restriction

Decreased birth weight of children from mothers smoking during pregnancy has been described since the late 1950's [27–30] and also occurs in prenatally exposed mice [31]. To identify vulnerable developmental windows, Esposito et al exposed pregnant mice to a combination of main- and sidestream smoke (meaning smoke generated from the filtered or burning end of a cigarette, respectively, thus mimicking active and passive smoking) during different periods of gestation. Smoking either only during the pre-implantation period, or throughout the entire pregnancy decreased fetal weight significantly, while fetal length was decreased only by exposure in early

pregnancy [32]. Another study also demonstrated sex-differences, as decreased body weight was observed in female but not male murine offspring after *in utero* smoke exposure [33]. These studies confirm that the influence of cigarette smoke on fetal growth observed in humans is largely reproducible in mice.

Hallmarks of asthma in prenatally exposed mice

Exposing pregnant mice to mainstream smoke from ED 8 to ED 20 significantly decreased lung volume and compliance, and increased airway resistance in two week old offspring compared to air controls. These findings led to the assumption that reduced body size and lung volume could be a main factor in driving the higher risk for respiratory diseases in later life [34].

Children growing up in smokers´ households tend to become smokers themselves [35], which could add to lung function loss initially induced by maternal smoking [36]. Further, maternal smoking appeared to aggravate effects of own smoking [36]. In line with human studies*, in utero* exposed mice had decreased lung compliance and volume, increased tissue elasticity and resistance. Additional exposure during adolescence and early adulthood from PND 21-49 not only aggravated lung function deficits, but further resulted in collagen deposition around the main bronchi [25]. The body weight of offspring exposed both *in utero* and during adolescence was significantly decreased compared to animals that were exposed to cigarette smoke *in utero* only. This study not only confirms the observations made in humans, but further suggests lasting structural changes of the airways when *in utero* exposure continues in early life [25].

As smoking during pregnancy does not only influence lung growth but also increased the risk for asthma during the lifespan [3–5], mouse models have been exploited to identify underlying mechanisms. Blacquiere et al combined *in utero* smoke with postnatal challenge with house dust mite (HDM), which led to enhanced goblet cell hyperplasia and mucus production. Furthermore, collagen III deposition around the airways was increased and correlated with increased stiffness of the airways and therefore a decrease in compliance [24]. In another study, enhanced methacholine (MCh) responsiveness was associated with a decrease in cyclic adenosine monophosphate (cAMP), which contributes to the relaxation of lung smooth muscle cells while its reduction led to airway hyperresponsiveness (AHR) [37]. Eyring and colleagues also confirmed the aggravation of allergic airway disease after *in utero* smoke exposure [38].

Taken together, the clinical findings seen in prenatally exposed children are reflected in mouse models, suggesting the usefulness of these models for mechanistic studies.

Influence of prenatal smoking on developmental pathways and epigenetic regulation

The Wingless Int-1 (Wnt)-β-catenin signaling pathway is critical for branching and morphogenesis of the fetal lung [39] and aberrant Wnt signaling has been related to asthma [40]. Prenatal smoking has been shown to reduce Wnt-related signaling such as Frizzled-7 (*Fzd7)* and β-catenin *(Ctnnb1)* and further the downstream Wnt target gene Fibronectin 1 (Fn1) in neonatal mice [41]. Additionally, factors involved in alveolarisation such as Forkhead-Box-Protein A2 (*Foxa2)* and Platelet-derived growth factor receptor alpha *(Pdgfra)* were decreased in the lungs of 1 day old offspring.

Alveolarisation was further investigated by addressing the retinoic acid (RA) signaling pathway. In 5 day old pups, several signaling molecules along this pathway such as RA receptor alpha (Rara), RA receptor beta (Rarb), retinoid-X receptor alpha (Rxra), retinaldehyde dehydrogenase-1 (Raldh1), cytochrome P450 26b1 (Cyp26b1) and nuclear receptor family 2, group F, member 2 (Nr2f2) were significantly decreased at the mRNA level after *in utero* smoke exposure [42].

The transcription factor runt-related proteins (RUNX) [43] is expressed in human and murine lungs in the pseudoglandular and canalicular stages. This transcription factor family is further involved in immune function and regulation. RUNX1 and RUNX3 in particular play a role in the maturation of thymocytes: RUNX1 suppresses the expression of cluster of differentiation 4 (CD4) in double-negative precursor T cells and RUNX3 is involved in differentiation and functionality of $CD8^+$ T cells [44,45]. *In utero* smoke exposure decreased the expression of RUNX1 and RUNX3 in the developing lung at PND 3 and PND 5 suggesting them as mediators both of deregulated immunity and increased risk of developing asthma [46].

A recent study examined the correlation of the insulin-like growth factor (IGF) axis and abnormalities in body weight, highlighting IGF-1 and IGF-1 receptor (IGF-1R). The authors investigated 30-day-old offspring that were prenatally exposed to cigarette smoke and observed a sex-dependent effect on body weight (only in females), which was accompanied by a decline of mRNA expression of IGF-1 and IGF-1R. Further, epigenetic modifications were suggested by DNA methylation of the IGF-1R promoter region. Meyer et al concluded that the changes in IGF-1 and IGF-1R expression, growth restriction and alterations in DNA methylation can lead to higher asthma susceptibility later in life due to the involvement of the IGF axis in lung development [33]. This is of interest as epidemiology studies show that alterations in DNA methylation are associated with increased asthma risk later in

life [47–49]. The abovementioned study was recently confirmed by Dehmel and co-authors. In brief, they identified IGF-1 to be decreased in fetal lungs (E 18.5) after *in utero* cigarette smoke exposure, which was associated with a fetal and postnatal growth retardation and a loss in lung function in females (at PND 21) [50]. Altogether, the results of these studies demonstrate that effects induced by *in utero* smoke exposure go far beyond the neonatal period and that these changes appear to affect male and female offspring in a different manner.

Models of sidestream cigarette smoke exposure during pregnancy

As prohibition of smoking in public places is incomplete in many countries [51], pregnant women can be exposed to environmental tobacco smoke ((ETS), experimentally corresponding to side-stream smoke) at public places as well as at home. ETS is a known risk factor for asthma (reviewed in [52]) and lower birth weight [53–55] which is also seen in mice [56,57].

Hallmarks of asthma in mice prenatally exposed to ETS

Ten years ago a hallmark study by Penn et al demonstrated that *in utero* ETS exposure exacerbates responsiveness to allergens [58] which has been confirmed in several studies by that group since then [26,59]. For example, *in utero* ETS exposure followed by postnatal allergen challenge with ovalbumin (OVA) increased T helper cells type 2 (Th2) cytokines in bronchoalveolar lavage fluid (BALF) and lung, lung eosinophilia, OVA-specific immunoglobulin (Ig)E and AHR [59]. Interestingly, these findings were more pronounced in male offspring.

Exposure to ETS *in utero* usually continues postnatally. To mimic this situation, Sing et al used following experimental setup: male and female animals of the F0 generation were adapted to ETS or filtered air for two weeks prior to mating. Smoking was then continued in females during pregnancy and - together with their pups - until three weeks after birth.

Subsequent challenge of offspring with *Aspergillus fumigatus (A. fumigatus)* induced a mixed eosino-neutrophilic airway inflammation and enhanced airway resistance and Th2 cytokines in ETS exposed animals. These changes correlated with higher lung expression of M1, M2, and M3 muscarinic receptors and phosphodiesterase-4D5 (PDE4D5) isozyme. The PDE4-selective inhibitor rolipram attenuated AHR, muscarinic receptors and PDE4D5, but left lung inflammation, Th2 cytokines or serum IgE levels unaffected [60]. While this model shows the sensitivity of the fetus to ETS, a paternal influence cannot be excluded with certainty: for example ETS exposure could alter sperm cell microRNA (miRNA) signatures which in turn might influence early gene expression programs in the early zygote. In fact, this has already been demonstrated in a model of chronic paternal stress where the father's phenotype was transmitted this way [61].

Enhanced airway and immune responses after prenatal ETS exposure is independent from the type of allergen, as provocation with HDM allergen of adult offspring increased airway inflammation, Th2 cytokines and induced remodeling similar to the *A. fumigatus* model [62].

Several investigators have already tried to pin down the susceptible developmental windows critical for exposures [60,63–66]. In the ETS *A. fumigatus* model described above, pre- but not postnatal ETS exacerbated AHR, levels of Th2 cytokines and atopy. Both prenatal

and/or postnatal ETS downregulated the T helper cells type 1 (Th1)-specific transcription factor T-bet and - despite high levels of interleukin (IL-)4 / IL-13 - blocked the Aspergillus-induced goblet cell metaplasia and mucus production regulating molecules (Mucin 5ac (*Muc5ac)*, Type A gamma-aminobutyric acid (GABAA)-receptors, and SAM pointed domain-containing ETS transcription factor (Spdef)). This led to the speculation that *in utero* exposure to ETS not only enhances characteristics of asthma, but also impairs lung development leading to reduced mucociliary clearance and Th1 responses [65]. Aside from prenatal exposure, early life may also be critical as early postnatal ETS similarly reduced lung function [63].

In another study, *in utero* ETS exposure was continued in male offspring until adulthood: It induced enlarged alveolar spaces and vessels independent of whether or not this was combined with exposure during adulthood. Additional exposure during adulthood induced vascular remodeling, and changed breathing patterns indicative of AHR accompanied by upregulation of transcripts of the metalloproteases a disintegrin and metalloproteinase with thrombospondin motifs 9 *(Adamts9)* and matrix metalloproteinase 3 *(Mmp3)* [66]. The observed pulmonary morphological changes could further indicate a higher susceptibility for COPD [66]. This contention is supported by a study showing that *in utero* ETS exposure alone can be sufficient to induce lung structural changes [67].

Influence of ETS on molecular pathways and epigenetic regulation

So far, mechanistic studies investigating the influence of prenatal ETS on the developing organism are scarce. Exposure to ETS during pregnancy leads to a deregulation of the placental and umbilical cord blood transcriptome in humans [68]. this line, a four day exposure to ETS in the last trimester of

murine pregnancy decreased placental and fetal weights, which was accompanied by decreased placental activation of the mechanistic Target of Rapamycin (mTOR) family of proteins which are involved in regulation of cell growth via nutrient sensing [56]. Another study demonstrated an increase of substance P airway innervation, neuropeptide Y (NPY) protein levels and density of NPY fibers in the lung, as well as pulmonary levels of nerve growth factor (NGF) later in life after prenatal and early postnatal ETS, which was paralleled by lower lung function [63,69]. ETS exposure in adolescent mice did not produce these changes suggesting altered pulmonary innervation developing in response to ETS in early life only [63].

Further, HDM-induced allergic inflammatory responses were aggravated after prenatal ETS, which coincided with lower global methylation in lung, spleen, and blood DNA. In addition, gene-specific analyses revealed strong hypo-methylation of *Il4* and *Il13*, while interferon gamma (*Ifng)* and forkhead box P3 (*Foxp3)* were hyper-methylated in double exposed animals [62]. Xiao and colleagues investigated miRNAs as another layer of epigenetic regulation and found among nine up-regulated miRNAs, miR-155-5p, miR-21-3p, and miR-18a-5p being highly correlated with pro-asthmatic Th2 cytokine levels in BALF of ETS exposed and OVA challenged animals [70]. Further analysis revealed that these up-regulated miRNAs shared common chromosome locations with pro-asthmatic genes suggesting joint regulation.

In conclusion, there are strong indications in current literature that the asthma risk of the offspring can also be determined by ETS, which underlines the importance of smoke prohibitions i.e. in the work environment and public places.

Prenatal smoking and phenotypes beyond the F1 generation

The postulate that grandmaternal smoking during pregnancy affects the propensity of her grandchildren to develop asthma is challenging to assess in humans. In rodent animal models, there are to our knowledge only two studies going beyond the F1 generation.

In the first study, Rehan et al treated pregnant rats with nicotine subcutaneously and observed alterations in lung mechanics of F1 and F2 offspring compared to controls. Moreover, the F1 generation had globally increased acetylation of histone 3 (H3) in the lung and decreased H4 acetylation and DNA methylation in the testis, whereas H4 acetylation was increased in the ovaries thus providing for the first time mechanistic insight underlying multigenerational transmission of asthma risk [71]. The investigators moved on to the F3 generation - which is the first generation not being exposed via the grandmother - and observed increased pulmonary resistance and decreased compliance in both sexes [72]. Of note, AHR in response to acetylcholine was only seen in males of the F2 and F3 generations [71,72].

In a second study, Singh et al exposed adult male and female mice to ETS two weeks prior mating and continued ETS-exposure until dams gave birth. *In utero* ETS-treated, and postnatally *A. fumigatus*-challenged F1 offspring revealed altered lung function with increased eosinophilia and Il-13 levels, whereas interferon gamma (INF-γ) levels were decreased in BALF compared to air-exposed controls. Of note, these effects were transmitted to the F2 generation. The authors found a decrease in hypoxia inducible factor 1 alpha (HIF-1α), NFκB and RUNX3 in lungs from seven day old F1 or F2 generation offspring. Due to a persistent pulmonary deregulation of microRNAs known to regulate angiogenesis and apoptosis, miR130, miR-16 and miR-221, the authors suggested epigenetic mechanisms underlying the transgenerational inheritance of exacerbated allergic asthma and bronchopulmonary dysplasia (BPD).[57]

Limitations of murine models and current knowledge gaps

In summary, murine models largely reproduce the findings on prenatal smoking obtained from epidemiological studies and have proven to be very useful for the investigation of underlying mechanisms. As explained above, most models depict phenotypic alterations in offspring such as *in utero* growth restriction, airway inflammation and airflow limitation. While these studies are encouraging, there are several limitations of murine transgenerational smoke exposure models that need to be kept in mind:

Most published studies differ remarkably regarding exposure systems, the timing and quantification of exposures, or the utilization of filtered or non-filtered cigarettes. At the same time objective measurements of cigarette smoke exposure are often insufficiently described. Along this line, it is crucial to assess the quantification of particles for every exposure setting, thus allowing a better comparison among different studies. Further, it is very difficult to control for maternal stress. The pure smell of smoke most likely puts the animals into stress that cannot be mimicked by simply handling animals that are exposed to air the same way as smoke exposed animals. In fact, stressing pregnant mice with noise has been shown to enhance asthma susceptibility in offspring [73]. Additionally, smoke-exposed mice often have a lower weight gain during pregnancy when compared to air controls, which may further confound the results. In addition, also the routes of smoke exposure may influence the resulting phenotype. For example, pregnant mice are usually exposed in whole body chambers, leading to the deposition of smoke particles in fur, which are ingested upon grooming. Last but not least, multigenerational studies up to the F2 and F3 generations are expensive, time consuming and need ample animal space.

Thus, there is a clear need for simpler animal models where maternal stress can be better controlled for and that allow faster progression into next generations.

Drosophila melanogaster

The fruit fly *Drosophila melanogaster* is increasingly recognized as a suitable non-vertebrate model system for asthma research [74,75] as it allows studying abnormalities of the airway epithelial barrier function and innate immunity that are typical of the disease [76] Even if the respiratory system of the fruit fly is only an analogue organ to the human lung, both systems have much more in common than anticipated at first sight. In both species, their airways are made of a tubular network that repeatedly branches from proximal to distal where it mediates the gas exchange [77] (**Fig. 2E and 2F**). However, unlike to mammals, flies do not breathe actively. Air enters the respiratory system through breathings openings (so-called spiracles) from where it is distributed in the entire airways by passive diffusion. When comparing the average particle size distribution of mainstream tobacco smoke (0.3 to 0.5 µm) with the average diameter of the smallest branches, called tracheoles (1.0 µm), smoke particles can easily enter the entire airway tree. Thus, even after a short exposure, the concentration of smoke in the respiratory system will be equal to that in the chamber of the smoking machine [78].

Like in human, all branches of the fly's airways are solely covered by a single epithelial layer with each cell along the airway tract being immunoreactive and acts as an innate immune system [79]. It has to be noted that drosophila lacks an adaptive immune system and some innate immune cells like eosinophils and mast cells, as well as structural cells such as smooth muscle cells and fibroblasts. This needs to be taken into account when using drosophila for asthma research, as all of those cell types play a pivotal role in the pathologenesis of asthma in patients. On the other hand, *Drosophila melanogaster* can thus be used to study epithelial contributions to pathology without confounding

effects by other cells. Moreover, the fly's airway epithelium shares many genes and molecular pathways (such as Toll like receptor (TLR)/IL1; TNFα signaling) with human ones [80]. These features of the fly's airway epithelium facilitate the identification and analysis of genes and mechanisms involved in (1) epithelial immune responses, (2) remodeling processes and (3) the maintenance of barrier integrity. Given the key role of the airway epithelium in asthma pathogenesis [81,82], the fly can be primarily used to investigate molecular aspects of airway remodeling initiated by the epithelium itself. In addition, it can be utilized to evaluate the functional role of asthma susceptibility genes in the development of the disease [83]. As most of these genes have at least one counterpart in the fly's genome and are also expressed in its airways [83,84], gene-environment interactions can be studied easily by manipulating genes of interest exclusively in airway epithelial cells. By subsequent exposure to common risk factors for asthma, gene-environment interactions can be comparatively easily assessed by determining the developmental time or the degree of airway branching (i.e. number and length) in particular. This is exemplified by the work of Kallsen et al who used the fly to obtain first insight into the function of the gene orosomucoid 1-like 3 (ORMDL3). Polymorphisms in this gene are strongly associated with childhood asthma [85]. The authors showed that enhanced epithelial expression of the *Drosophila* ORDML homologue reduces the ability of adult flies to cope with asthma risk factors such as cigarette smoke [75].

To the knowledge of the authors, a *Drosophila* model to investigate the airway response to cigarette smoke exposure has not been reported so far. Even though there are a number of publications that address the toxicological effects of cigarette smoke and nicotine on the fly's health, none of these publications aimed at establishing a *Drosophila* model for cigarette smoke exposure reflecting central characteristics of an antioxidant or xenobiotic response. This is certainly a major reason why *Drosophila* has not yet been utilized for studies on transgenerational inheritance and why there is a lack of experimental evidence showing that cigarette smoke-induced transgenerational alterations

are regulated similarly between fly, mouse, and human. Nonetheless, there is evidence that pathways associated with the response to cigarette smoke are conserved between species, as for example Cytochrome P450 (CYP) analogs. In the fruit fly, there are 83 CYP genes, while most of their functions are not fully elucidated so far. Recent research shows that they are primarily involved in developmental processes and in the detoxification of xenobiotics. In this respect it's known that Cyp6g1 confers nicotine resistance [86]. A further promising candidate might be Cyp18a1 as its human homolog Cyp1a1 contributes to the detoxification of polycyclic aromatic hydrocarbons (PAHs) found in the tar fraction of cigarette smoke.

Of note, using *Drosophila* offers several advantages for studying transgenerational inheritance: first of all, transgenerational studies on F3 and subsequent generations can be performed quite quickly due to its short generation time (about 9 days at 25°C) (**Fig. 1B**); second, it can be cultured easily, inexpensively and in large numbers due to its high reproductive rate, and third, one can proceed faster from inter- to transgenerational studies as fly embryogenesis occurs outside of the female body whereby the offspring is not subject to the "lifestyle" of the grandmother (reviewed in [87]). In fact, transgenerational *Drosophila* models of diet-induced obesity have already been described as a powerful tool to identify and characterize molecular mechanisms underlying transgenerational transmission of metabolic traits [88,89]. In these studies, obese female flies produced solely offspring with an obese-like phenotype even though the offspring were raised on a standard fly diet for their entire life cycle. Interestingly, this phenotype was transmitted for both sexes into the third generation, demonstrating a transgenerational transmission of maternal obesity in *Drosophila* [88].

Danio rerio

Zebrafish are small, tropical fish which are easily and inexpensively maintained in laboratory conditions [90]. The fertilization and embryonic development of zebrafish occurs extra-corporally, facilitating parent-of-origins studies while minimizing the influence of maternal stress related to the exposure of interest. Zebrafish reach sexual maturity within 2-3 months and produce large numbers of offspring in one breeding (100-200 embryos per pair). The embryos develop in water and are transparent, which facilitates exposure, imaging and morphological analyses. All major organs are developed very fast within three days post fertilization (**Fig. 1C**) [91], thus enabling to investigate the influence of cigarette smoke from the moment of fertilization throughout the entire development. The whole genome of zebrafish is sequenced with around 70% similarity to humans while 84% of zebrafish genes have counterparts in human diseases [92]. Additionally, there are many knock-out, transgenic and reporter zebrafish lines available which enables not only the dissection of developmental patterns, but also to decipher the molecular mechanisms underlying human diseases. Also, laboratory zebrafish are less inbred and more genetically diverse compared to rodents, in this way being more similar to human populations. All these factors indicate that zebrafish are a suitable model to study transgenerational inheritance of disease risk.

Similar to *Drosophila*, zebrafish have only recently been introduced into respiratory research [93]. One obvious reason for this could be that zebrafish do not have lungs. Nonetheless, gills and swim bladder can both be used for lung research due to their evolutionary similarities (**Fig. 2G and 2H**). Gills have the same gas-exchange function as lungs (mucus-covered respiratory epithelium scattered with immune cells and smooth muscle cells at the base of the lamella) [93]. The swim bladder has the same embryonic background as the mammalian lung, it is covered with surfactant and its transcriptome is comparable to that of the lung [94]. It is lined by a single layer of epithelial cells, with scattered rodlet cells (presumed to be counterparts of mast/eosinophil cells) and a basement

membrane. The structures bellow this epithelial layer have some differences depending on the regional part of the swim bladder [95]. The lateral posterior chamber has four distinct layers: squamous epithelium with a basement membrane, a lamina propria consisting of connective tissue matrix with collagen and elastin fibers, a muscular mucosa containing layers of smooth muscle cells and scattered fibroblasts, and connective tissue with blood vessels and nerves [96]. It has recently been shown that the swim bladder can be used as a model for acute lung injury. The injection of LPS into the swim bladder leads to similar effects that are characteristic for acute lung injury in humans: neutrophil infiltration and recruitment, increase in inflammatory cytokine expression, and *in situ* injuries, including epithelial distortion, endoplasmic reticulum swelling and mitochondrial injuries [97]. Further, several signaling pathways involved in human lung development, such as sonic hedgehog signaling and Wnt/β-catenin signaling pathways, are also essential for zebrafish swim bladder development [98,99]. As mentioned above, Wnt signaling is an important contributor to the transgenerational inheritance of smoking effects, while β-catenin and frizzled7b (Fz7b) are also important for swim bladder formation in zebrafish[98]. However, to our knowledge there is no data on their expression under the influence of cigarette smoke extract (CSE) or nicotine in zebrafish. A recently published study shows that exposure of zebrafish larvae to total particulate matter (TPM) from cigarette smoke after the 72 hours post fertilization (hpf) exposure have significantly decreased total body β-catenin protein levels [100], which is a hint for a similar regulation as in mouse models.

Further, it should be noted that, with some differences, also the zebrafish xenobiotic metabolism is comparable to that of humans [101]. There are 94 CYP genes identified in zebrafish, divided into 18 gene families [102]. The genes involved in drug and xenobiotic metabolism are mostly members of families 1 to 4. Zebrafish Cytochrome P450, family 1 (CYP1) genes are distributed into four subfamilies (A-D) and have been cloned and sequenced. Exon structure of zebrafish Cytochrome P450, family 1, member A (CYP1A) gene is similar to human CYP1A1 and CYP1A2 genes. CYP1A is

inducible by AHR agonists, as well as by exposure to oxidative stress and ultraviolet (UV) radiation [101]. The expression of the CYP1A1 gene is highest in the adult liver, gut tissue, abdominal cavity and cardiovascular system during embryonic development, with a peak of expression after hatching (72 hpf) [103]. There is a broad spectrum of substrates that are metabolized by both human and zebrafish CYP1. CYP1A is the most active CYP in zebrafish and it metabolizes 7-ethoxyresorufin (ER), 7-benzyloxyresorufin (BR) and 7-methoxyresorufin (MR) similar to mammalian CYP1A1 [104]. It is the dominant CYP responsible for ER metabolism. The measurement of CYP1A activity can be done *in vivo* in zebrafish using 7-ethoxy-resorufin-O-deethylase (EROD) assay [105]. It has been shown that the expression of CYP1A in zebrafish embryos is upregulated after the exposure to CSE [106], similar to what happens in humans and mice. However, in another study, the activity of CYP1A was shown to be decreased after the exposure to TPM, but this could be due to the embryonic stage when the activity was measured [100].

It has to be noted that zebrafish can obviously not inhale cigarette smoke, but the exposure of the whole body occurs in water through the skin. Usually, the zebrafish embryos are exposed to various doses of CSE, TPM or specific cigarette smoke components, such as nicotine, at different time points and observed for different effects during development (from fertilization to 120 hpf). A great advantage of zebrafish is their extracorporeal development, enabling easy monitoring and studying specific effects on the organ of interest by adjusting the period of exposure. Further, the influence can be investigated in several other stages – during the development of ovaries and/or testicles, which happens in week three and seven post fertilization, respectively (**Fig. 1C**) – and the exposure of adult fish is possible [107]. In the literature, there are limited data about the effective doses of cigarette smoke extracts. One study showed that the doses causing toxic effects on zebrafish embryos are in small range of concentrations, that the effects largely overlap and are comparable to those that are caused by smoking during pregnancy (body axis malformations, head and trunk

abnormalities and significant developmental delay) [106]. LD50 (lethal dose) for CSE of different brands of cigarettes varied from 25 to 50 µg/mL, and doses between 20 and 30 µg/mL having prominent effects on developmental malformations [106]. Regarding a sole nicotine exposure, it has been shown that with constant nicotine concentration in water, the levels of nicotine in zebrafish plasma are constant, mimicking levels found in smokers individuals using nicotine patches [108]. Thus, even if not all ingredients of cigarette smoke are soluble in water, zebrafish could present a good model to investigate the effect of single compounds of cigarette smoke and their defined combinations during specific phases of embryonic development.

As mentioned above, there are several published studies addressing the effects of CSE on zebrafish development [93,106,109–111]. All of these studies found developmental adverse effects when zebrafish embryos were exposed to CSE. In all cases, the effects of CSE were more toxic than single exposure to nicotine in equivalent doses. Studies so far predominantly explored toxicological effect on morphology and behavior, and little attention was given to molecular effects of CSE. One recent study investigated the use of gills as a model for studying immune responses in chronic lung diseases, such as asthma, COPD and idiopathic lung fibrosis [93]. The exposure of adult fish to CSE increased the expression of pro-inflammatory cytokines tumor necrosis factor alpha (TNFα), IL-1β and matrix metallopeptidase 9 (MMP9) in the gills, which was not the case when acrolein or nicotine alone was used. After chronic exposure to CSE for 6 weeks, the gills developed structural changes without signs of inflammation or fibrosis [93]. Additionally, numbers of gill neutrophils were decreased, which was attributed to increased apoptosis or formation of neutrophil extracellular traps (NETosis).

In the study of Massarsky and colleagues, embryos were exposed to different doses of nicotine alone or TPM extracts. Embryos exposed to nicotine alone did not vary significantly from the control group. On the contrary, embryos that were exposed to the doses of TPM equivalent to those of nicotine had an increase in developmental deformities, accompanied by delayed hatching, increased mortality, reduced body length and heart rate. The activity of CYP1A1 was decreased and of glutathione-S-transferase increased after the exposure [109]. Ellis and colleagues monitored the acute and developmental effects of CSE as well as the effects on behavior, and showed that different cigarette brands led to different outcomes, with some overlaps. They found similar effects to those reported in the Massarsky study and showed altered expression of genes involved in xenobiotic metabolism (cyp1a1, cyp1b1, cyp2aa12, ahrr1), apoptosis (heat shock protein 70 (hsp70), growth arrest and DNA-damage-inducible, alpha, a (gadd45al)), pigment formation (cystathionine-beta-synthase b (Cbsb), microphthalmia-associated transcription factor a (Mitfa)) and regulatory genes (p21 protein (Cdc42/Rac)-activated kinase 2a (pak2), Kruppel-like factor 2a (klf2a)) [106]. Others studied the effects of CSE and e-cigarette aerosol on zebrafish cardiac development. They showed that both exposures had negative effects on cardiac development, but that the cigarette smoke was more toxic and had a broader spectrum of cardiac developmental defects [110]. Taken together, these studies demonstrate that zebrafish is a useful model for testing the effects of water-soluble CSE on development and could provide a greater insight into the mechanisms by which cigarette smoke and its components establish their deleterious effects.

As mentioned before, zebrafish can efficiently be used in transgenerational studies. Transgenerational inheritance in zebrafish has been shown in studies of hypoxia, dioxin, bisphenol A (BPA), hormones and a range of environmental pollutants [112–115]. To the authors' knowledge, there are no transgenerational studies on zebrafish on the effects of cigarette smoke, but one study addressed effects of nicotine on the F1 generation. This study investigated the consequences of maternal acute or chronic nicotine exposure and observed that there was an alteration in expression of myelin-related genes in the offspring [107].

In summary, it has been shown that exposure to CSE/TPM causes growth retardation during embryogenesis, as well as changes in expression of several genes comparable to that of human and mouse. Further studies on other hallmarks of respiratory diseases in zebrafish are needed, but zebrafish is emerging as a valuable model for respiratory diseases and transgenerational research and could prove to be an inexpensive and practical model bridging cell culture and mammalian studies.

Conclusion

Taken together each of the models has specific advantages and disadvantages for studies across generations (summarized in Table 1). In a synergistic approach, benefit could be taken of the comparatively fast replication of *Drosophila melanogaster* and *Danio rerio* to identify signaling pathways and epigenetic changes involved in early programming of disease risks and their potential transmission across multiple generations (**Fig. 3**). Preselected and highly conserved pathways can thus be investigated in a focused manner and more efficient than in the more laborious mouse model. The latter - different from flies and fish - allows to test how these pathways affect the asthma phenotype. The mouse models further allows preclinical testing of interventions preventing deregulation of pathways involved in transgenerational transmission of disease risks. The proposed stepwise research strategy will hopefully identify new molecular targets and efficiently verify pre-identified molecular targets from human epidemiological and clinical studies.

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CONFLICT OF INTEREST STATEMENT

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Table 1 Characteristics of laboratory mouse, fly and fish in the context of transgenerational studies

	M. musculus	**D. melanogaster**	**D. rerio**
Immune system	Innate & adaptive	innate	Innate and adaptive
Lung & homologue	Mammalian lung	Tubular network of airways with branches; no alveoli	Gills and swim bladder
Lung (homologue) developmental stages clearly defined	Yes	Yes	Yes
Homology to human genome	85% [116]	60% [117]	70%
Maternal stress during embryonic development	Partly controllable	Partly controllable	Controllable
Extra corporal fertilization	Feasible but very laborious	Not possible	Always
F2 generation exposed via germ cells	yes	no	no
F1 generation exposed via mother	yes	yes	no
Multigenerational studies	Take several months	F3 reached within 3 weeks	F3 reached within 16-24 weeks
Paternal exposures	Need ample breeding space	Easy to perform	Easy to perform
Experimental asthma induction	Standard procedure for various allergens	Not possible	Not possible
Methylation analysis	Possible	Not possible	Possible
Histone modification analysis	Possible	Possible	Possible
miRNA analysis	Possible	Possible	Possible

FIGURE LEGENDS

Figure 1. Developmental stages of mouse, fruit fly and zebrafish. (A) *Mus musculus*: Starting with the conception, embryonic and fetal development occur until 19-21 days (d) of gestation. At birth (PND 0) the pups are blind, nude, the ears are closed and the toes are not spread. The offspring is weaned from the mother fully developed at PND 18-21 and starts puberty at 4 weeks of age. From 3rd to 8th week mice are defined as juvenile and are considered adult at the age of 8 weeks. **(B) *Drosophila melanogaster*:** Fruit flies are holometabolous insects that undergo metamorphosis. Their life cycle comprise four distinct stages: egg (embryo), larva (1st, 2nd, 3rd instar), pupa (prepupa/white pupa, brown pupa), and imago (adult). Embryo and larva stages could cautiously be translated as an intra-uterine environment and pupa as childhood and adolescence. The rate of development depends among others on temperature, being faster at higher temperatures. For example, at 20°C, the life cycle lasts 14 or 15 days, whereas at 25°C, it is completed not later than on day 10. AED: after egg deposition. **(C) *Danio Rerio*** *(adapted from* [118]*):* The zebrafish life cycle consists of four stages: embryo, larva, juvenile and adult. It takes around 10-12 weeks to develop from fertilized egg to adulthood. The first phases of development are very rapid and occur at predictable rate at 28°C. The increase or decrease in temperature changes the rate of early development, making it faster or slower, respectively.

Figure 2. The respiratory systems of human, mouse, drosophila and zebrafish. (A-B) The human lungs are located in the thorax, where the left lung is divided in two lobes and the right lung has three lung lobes (A) with a dichotomous airway branching (B). (C-D) The murine lungs consist of one left lung lobe and four right lung lobes (C) and its airways branch monopodial (D). (E-F) The respiratory (tracheal) system of Drosophila consists of a highly branched network of epithelial tubes and air sacs of varying size (dorso-lateral view; oriented anterior left). Air enters and leaves the tracheal system through the breathing openings (spiracles) located laterally along the thorax and

abdomen. While the head and thoracic air sacs serve for gas storage, the abdominal tracheal system primarily functions in gas transport and exchange. (F) The abdominal tracheal system ramifies from proximal to distal, where they end up in terminal endings, the so-called tracheoles. There, oxygen enters directly the tissue or open blood system (hemolymph) via diffusion, while carbon dioxide (CO_2) dissolved in the hemolymph are removed into the tracheal system in order to be excreted. (G-H) The zebrafish respiratory organs are the gills but the air-filled organ swim bladder can also be used as a model for lung development (G). The swim bladder is connected to the oesophagus via pneumatic duct by which it fills with air. Gas exchange occurs in the lamellae of gill filaments which are in contact with water that runs from mouth through pharynx and exits through the caudal opening of the operculum (H).

Figure 3. Proposed identification and selection strategy for pathways involved in early life programming of respiratory disease. Potential risk exposures are identified in children of human cohort studies. To identify the underlying mechanisms, zebrafish and fruit fly serve as models to investigate deregulated pathways and epigenetic modifications in short-time multigenerational studies. Observed alterations can be translated into mammalian models like the mouse to study resulting disease phenotypes in offspring. To confirm deregulated pathways, a validation in patient subgroups is needed.

References

1. Abel, E. L. Smoking during pregnancy: a review of effects on growth and development of offspring. *Hum Biol* **52,** 593–625 (1980).

2. Gibbs, K., Collaco, J. M. & McGrath-Morrow, S. A. Impact of tobacco smoke and nicotine exposure on lung development. *Chest* **149,** 552–561 (2016).

3. Stick, S. M., Burton, P. R., Gurrin, L., Sly, P. D. & LeSouef, P. N. Effects of maternal smoking during pregnancy and a family history of asthma on respiratory function in newborn infants. *Lancet* **348,** 1060–1064 (1996).

4. Gilliland, F. D., Li, Y. F. & Peters, J. M. Effects of maternal smoking during pregnancy and environmental tobacco smoke on asthma and wheezing in children. *Am J Respir Crit Care Med* **163,** 429–436 (2001).

5. Dai, X. *et al.* Early smoke exposure is associated with asthma and lung function deficits in adolescents. *J. Asthma* 1–8 (2016). doi:10.1080/02770903.2016.1253730

6. den Dekker, H. T. *et al.* Tobacco Smoke Exposure, Airway Resistance, and Asthma in School-age Children: The Generation R Study. *Chest* **148,** 607–617 (2015).

7. Harju, M., Keski-Nisula, L., Georgiadis, L. & Heinonen, S. Parental smoking and cessation during pregnancy and the risk of childhood asthma. *BMC Public Health* **16,** 428 (2016).

8. Vardavas, C. I. *et al.* The independent role of prenatal and postnatal exposure to active and passive smoking on the development of early wheeze in children. *Eur. Respir. J.* **48,** 115–24 (2016).

9. Foreman, M. G. *et al.* Early-Onset Chronic Obstructive Pulmonary Disease Is Associated with Female Sex, Maternal Factors, and African American Race in the COPDGene Study. *Am. J. Respir. Crit. Care Med.* **184,** 414–420 (2011).

10. Svanes, C. *et al.* Early life origins of chronic obstructive pulmonary disease. *Thorax* **65,** 14–20 (2010).

11. Perret, J. L. *et al.* Mother's smoking and complex lung function of offspring in middle age: A cohort study from childhood. *Respirology* **21,** 911–919 (2016).

12. Svanes, C. *et al.* Parental smoking in childhood and adult obstructive lung disease: results from the European Community Respiratory Health Survey. *Thorax* **59,** 295–302 (2004).

13. Rockhill, K. M. *et al.* Postpartum Smoking Relapse After Quitting During Pregnancy: Pregnancy Risk Assessment Monitoring System, 2000–2011. *J. Women's Heal.* **25,** 480–488 (2016).

14. Tong, V. T., Jones, J. R., Dietz, P. M., D'Angelo, D. & Bombard, J. M. Trends in smoking before, during, and after pregnancy - Pregnancy Risk Assessment Monitoring System (PRAMS), United States, 31 sites, 2000-2005. *MMWR. Surveill. Summ. Morb. Mortal. Wkly. report. Surveill. Summ. / CDC* **58,** 1–29 (2009).

15. Wagner, N. J., Camerota, M. & Propper, C. Prevalence and Perceptions of Electronic Cigarette Use during Pregnancy. *Matern. Child Health J.* (2017). doi:10.1007/s10995-016-2257-9

16. Spindel, E. R. & McEvoy, C. T. The role of nicotine in the effects of maternal smoking during pregnancy on lung development and childhood respiratory disease: Implications for dangers of e-cigarettes. *Am. J. Respir. Crit. Care Med.* **193,** 486–494 (2016).

17. Miller, L. L., Pembrey, M., Davey Smith, G., Northstone, K. & Golding, J. Is the growth of the fetus of a non-smoking mother influenced by the smoking of either grandmother while pregnant? *PLoS One* **9,** e86781 (2014).

18. Li, Y.-F., Langholz, B., Salam, M. T. & Gilliland, F. D. Maternal and grandmaternal smoking patterns are associated with early childhood asthma. *Chest* **127,** 1232–41 (2005).

19. Magnus, M. C. *et al.* Grandmother's smoking when pregnant with the mother and asthma in the grandchild: the Norwegian Mother and Child Cohort Study. *Thorax* **70,** 237–243 (2015).

20. Lodge, C. J. *et al.* Grandmaternal smoking increases asthma risk in grandchildren: a nationwide Swedish cohort. *Clin. Exp. Allergy* (2017). doi:10.1111/cea.13031

21. Miller, L. L., Henderson, J., Northstone, K., Pembrey, M. & Golding, J. Do grandmaternal smoking patterns influence the etiology of childhood asthma? *Chest* **145,** 1213–1218 (2014).

22. Svanes, C. *et al.* Father's environment before conception and asthma risk in his children: a multi-generation analysis of the Respiratory Health In Northern Europe study. *Int. J. Epidemiol.* 1–11 (2016). doi:10.1093/ije/dyw151

23. Warburton, D. Lung Organogenesis. *Curr Top Dev Biol.* **2153,** 73–158 (2012).

24. Blacquière, M. J. *et al.* Maternal smoking during pregnancy induces airway remodelling in mice offspring. *Eur. Respir. J.* **33,** 1133–40 (2009).

25. Drummond, D., Baravalle-einaudi, M., Lezmi, G. & Vibhushan, S. Combined Effects of in Utero and Adolescent Tobacco Smoke Exposure. **392,** 392–399 (2017).

26. Rouse, R. L., Boudreaux, M. J. & Penn, A. L. In utero environmental tobacco smoke exposure alters gene expression in lungs of adult BALB/c mice. *Env. Heal. Perspect* **115,** 1757–1766 (2007).

27. Simpson, W. J. A preliminary report on cigarette smoking and the incidence of prematurity. *Am. J. Obstet. Gynecol.* **73,** 808–815 (1957).

28. Butler, N. R., Goldstein, H. & Ross, E. M. Cigarette smoking in pregnancy: its influence on birth weight and perinatal mortality. *Br Med J* **2,** 127–130 (1972).

29. Sexton, M. & Hebel, J. R. A clinical trial of change in maternal smoking and its effect on birth weight. *JAMA* **251,** 911–915 (1984).

30. Ward, C., Lewis, S. & Coleman, T. Prevalence of maternal smoking and environmental tobacco smoke exposure during pregnancy and impact on birth weight: retrospective study using Millennium Cohort. *BMC Public Health* **7,** 81 (2007).

31. Seller, M. J. & Bnait, K. S. Effects of tobacco smoke inhalation on the developing mouse embryo and fetus. *Reprod Toxicol* **9,** 449–459 (1995).

32. Esposito, E. R., Horn, K. H., Greene, R. M. & Pisano, M. M. An animal model of cigarette smoke-induced in utero growth retardation. *Toxicology* **246,** 193–202 (2008).

33. Meyer, K. F. *et al.* Prenatal exposure to tobacco smoke sex dependently influences methylation and mRNA levels of the Igf axis in lungs of mouse offspring. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **312,** L542–L555 (2017).

34. Larcombe, A. N., Foong, R. E., Berry, L. J., Zosky, G. R. & Sly, P. D. In utero cigarette smoke exposure impairs somatic and lung growth in BALB/c mice. *Eur Respir J* **38,** 932–938 (2011).

35. Vuolo, M. & Staff, J. Parent and child cigarette use: a longitudinal, multigenerational study. *Pediatrics* **132,** e568-77 (2013).

36. Dratva, J. *et al.* Early Life Origins of Lung Ageing: Early Life Exposures and Lung Function Decline in Adulthood in Two European Cohorts Aged 28-73 Years. *PLoS One* **11,** e0145127 (2016).

37. Singh, S. P. *et al.* Prenatal cigarette smoke decreases lung cAMP and increases airway hyperresponsiveness. *Am J Respir Crit Care Med* **168,** 342–347 (2003).

38. Eyring, K. R., Pedersen, B. S., Yang, I. V & Schwartz, D. A. In Utero Cigarette Smoke Affects Allergic

Airway Disease But Does Not Alter the Lung Methylome. *PLoS One* **10,** e0144087 (2015).

39. De Langhe, S. P. *et al.* Dickkopf-1 (DKK1) reveals that fibronectin is a major target of Wnt signaling in branching morphogenesis of the mouse embryonic lung. *Dev. Biol.* **277,** 316–331 (2005).

40. Sharma, S. *et al.* A role for wnt signaling genes in the pathogenesis of impaired lung function in asthma. *Am. J. Respir. Crit. Care Med.* **181,** 328–336 (2010).

41. Blacquière, M. J. *et al.* Maternal smoking during pregnancy decreases Wnt signalling in neonatal mice. *Thorax* **65,** 553–4 (2010).

42. Manoli, S. E. *et al.* Maternal smoking and the retinoid pathway in the developing lung. *Respir Res* **13,** 42 (2012).

43. Appleford, P. J. & Woollard, A. RUNX genes find a niche in stem cell biology. *J Cell Biochem* **108,** 14–21 (2009).

44. Naoe, Y. *et al.* Repression of interleukin-4 in T helper type 1 cells by Runx/Cbf beta binding to the Il4 silencer. *J Exp Med* **204,** 1749–1755 (2007).

45. Taniuchi, I. *et al.* Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell* **111,** 621–633 (2002).

46. Haley, K. J. *et al.* RUNX transcription factors: association with pediatric asthma and modulated by maternal smoking. *Am J Physiol Lung Cell Mol Physiol* **301,** L693-701 (2011).

47. Breton, C. V. *et al.* Prenatal tobacco smoke exposure is associated with childhood DNA CpG methylation. *PLoS One* **9,** (2014).

48. Joubert, B. R. *et al.* 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. *Environ. Health Perspect.* **120,** 1425–31 (2012).

49. Ladd-Acosta C, Shu C, Lee BK, Gidaya N, Singer A, Schieve LA, S. DE, 457 Jones N, Daniels JL, Windham GC, Newschaffer CJ, Croen LA, F. A., 20 & M., D. F. Presence of an epigenetic signature of prenatal cigarette smoke exposure in childhood. *Environ. Res.* **144,** 139–148 (2016).

50. Dehmel, S. *et al.* Intrauterine smoke exposure deregulates lung function, pulmonary transcriptomes, and in particular insulin-like growth factor (IGF)-1 in a sex-specific manner. *Sci. Rep.* **8,** 7547 (2018).

51. List of smoking bans. (2017).

52. JA. Castro-Rodriguez, E. Forno E, CE. Rodriguez-Martinez, J. C. Risk and Protective Factors for Childhood Asthma: What Is the Evidence? *J Allergy Clin Immunol Pr.* **4,** 1111–1122 (2016).

53. Hannah Burke *et al.* Prenatal and Passive Smoke Exposure and Incidence of Asthma and Wheeze: Systematic Review and Meta-analysis. *Pediatrics* **130,** S9–S9 (2012).

54. Subramoney, S., Espaignet, E. T. D. & Gupta, P. C. Higher risk of stillbirth among lower and middle income women who do not use tobacco , but live with smokers. 572–577 (2010). doi:10.3109/00016341003801656

55. Wahabi, H. A. *et al.* Effects of secondhand smoke on the birth weight of term infants and the demographic profile of Saudi exposed women. *BMC Public Health* (2013).

56. Mejia, C. *et al.* Decreased activation of placental mTOR family members is associated with the induction of intrauterine growth restriction by secondhand smoke in the mouse. *Cell Tissue Res.* 387–395 (2017). doi:10.1007/s00441-016-2496-5

57. Singh, S. P. *et al.* Gestational Exposure to Sidestream (Secondhand) Cigarette Smoke Promotes Transgenerational Epigenetic Transmission of Exacerbated Allergic Asthma and Bronchopulmonary

Dysplasia. *J. Immunol.* **198,** 3815–3822 (2017).

58. Penn, A. L. *et al.* In utero exposure to environmental tobacco smoke potentiates adult responses to allergen in BALB/c mice. *Env. Heal. Perspect* **115,** 548–555 (2007).

59. Xiao, R. *et al.* In utero exposure to second-hand smoke aggravates the response to ovalbumin in adult mice. *Am. J. Respir. Cell Mol. Biol.* **49,** 1102–1109 (2013).

60. Singh, S. P. *et al.* Maternal exposure to secondhand cigarette smoke primes the lung for induction of phosphodiesterase-4D5 isozyme and exacerbated Th2 responses: rolipram attenuates the airway hyperreactivity and muscarinic receptor expression but not lung inflammation and a. *J Immunol* **183,** 2115–2121 (2009).

61. Rodgers, A. B., Morgan, C. P., Leu, N. A. & Bale, T. L. Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. *Proc. Natl. Acad. Sci.* **2015,** 1–6 (2015).

62. Christensen, S. *et al.* Prenatal environmental tobacco smoke exposure increases allergic asthma risk with methylation changes in mice. *Environ. Mol. Mutagen.* **58,** 423–433 (2017).

63. Wu, Z. X. *et al.* Prenatal and early, but not late, postnatal exposure of mice to sidestream tobacco smoke increases airway hyperresponsiveness later in life. *Env. Heal. Perspect* **117,** 1434–1440 (2009).

64. Xiao, R. *et al.* In utero exposure to second-hand smoke aggravates adult responses to irritants adult second-hand smoke. *Am. J. Respir. Cell Mol. Biol.* **47,** 843–851 (2012).

65. Singh, S. P. *et al.* Prenatal secondhand cigarette smoke promotes Th2 polarization and impairs goblet cell differentiation and airway mucus formation. *J. Immunol.* **187,** 4542–52 (2011).

66. Xiao, R. *et al.* In utero exposure to second-hand smoke aggravates adult responses to irritants: adult second-hand smoke. *Am J Respir Cell Mol Biol* **47,** 843–851 (2012).

67. Noël, A. *et al.* Sex-specific lung functional changes in adult mice exposed only to second-hand smoke in utero. 1–12 (2017). doi:10.1186/s12931-017-0591-0

68. Votavova, H. *et al.* Deregulation of Gene Expression Induced by Environmental Tobacco Smoke Exposure in Pregnancy. **14,** 1073–1082 (2012).

69. Z.-X. Wu, K. B. Benders, D. D. Hunter, R. D. D. Early postnatal exposure of mice to side-steam tobacco smoke increases neuropeptide Y in lung. *Am J Physiol Lung Cell Mol Physiol.* **302,** L152–L159 (2012).

70. Xiao, R., No, A., Perveen, Z. & Penn, A. L. In Utero Exposure to Second-Hand Smoke Activates Pro-Asthmatic and Oncogenic miRNAs in Adult Asthmatic Mice. **199,** 190–199 (2016).

71. Rehan, V. K. *et al.* Perinatal nicotine exposure induces asthma in second generation offspring. *BMC Med* **10,** 129 (2012).

72. Rehan, V. K., Liu, J., Sakurai, R. & Torday, J. S. Perinatal nicotine-induced transgenerational asthma. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **305,** L501-7 (2013).

73. Pincus-Knackstedt, M. K. *et al.* Prenatal Stress Enhances Susceptibility of Murine Adult Offspring toward Airway Inflammation. *J. Immunol.* **177,** (2006).

74. Roeder, T., Isermann, K., Kallsen, K., Uliczka, K. & Wagner, C. A Drosophila asthma model - what the fly tells us about inflammatory diseases of the lung. *Adv. Exp. Med. Biol.* **710,** 37–47 (2012).

75. Kallsen, K. *et al.* ORMDL deregulation increases stress responses and modulates repair pathways in Drosophila airways. *J Allergy Clin Immunol* **136,** 1105–1108 (2015).

76. Loxham, M. & Davies, D. E. Phenotypic and genetic aspects of epithelial barrier function in asthmatic patients. *J. Allergy Clin. Immunol.* **139,** 1736–1751 (2017).

77. Rühle, H. *Das larvale Tracheensystem von Drosophila melanogaster Meigen und seine Variabilität*. (Akad. Verlagsges., 1932).

78. Snyder, G. K., Sheafor, B., Scholnick, D. & Farrelly, C. Gas exchange in the insect tracheal system. *J. Theor. Biol.* **172,** 199–207 (1995).

79. Wagner, C., Isermann, K., Fehrenbach, H. & Roeder, T. Molecular architecture of the fruit fly's airway epithelial immune system. *BMC Genomics* **9,** 446 (2008).

80. Bergman, P., Seyedoleslami Esfahani, S. & Engström, Y. in *Current Topics in Developmental Biology* **121,** 29–81 (Academic Press, 2017).

81. Lambrecht, B. N. & Hammad, H. Asthma: the importance of dysregulated barrier immunity. *Eur. J. Immunol.* **43,** 3125–37 (2013).

82. Holgate, S. T. Epithelium dysfunction in asthma. *Journal of Allergy and Clinical Immunology* **120,** 1233–1244 (2007).

83. Roeder, T., Isermann, K. & Kabesch, M. Drosophila in asthma research. *Am J Respir Crit Care Med* **179,** 979–983 (2009).

84. Chintapalli, V. R., Wang, J. & Dow, J. A. Using FlyAtlas to identify better Drosophila melanogaster models of human disease. *Nat Genet* **39,** 715–720 (2007).

85. Moffatt, M. F. *et al.* Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature* **448,** 470–473 (2007).

86. Li, X., Bai, S. & Cass, B. N. Accord insertion in the 5' flanking region of CYP6G1 confers nicotine resistance in Drosophila melanogaster. *Gene* **502,** 1–8 (2012).

87. Krauss-Etschmann, S., Meyer, K. F., Dehmel, S. & Hylkema, M. N. Inter- and transgenerational epigenetic inheritance: evidence in asthma and COPD? *Clin. Epigenetics* **7,** 53 (2015).

88. Buescher, J. L. *et al.* Evidence for transgenerational metabolic programming in Drosophila. *Dis Model Mech* **6,** 1123–1132 (2013).

89. Brookheart, R. T. & Duncan, J. G. Drosophila melanogaster: An emerging model of transgenerational effects of maternal obesity. *Mol Cell Endocrinol* (2015). doi:10.1016/j.mce.2015.12.003

90. Guo, S. Linking genes to brain, behavior and neurological diseases: what can we learn from zebrafish? *Genes Brain Behav* **3,** 63–74 (2004).

91. Westerfield, M. *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio Rerio)*. (University of Oregon Press, 2007).

92. Howe, K. *et al.* The zebrafish reference genome sequence and its relationship to the human genome. *Nature* **496,** 498–503 (2013).

93. Progatzky, F., Cook, H. T., Lamb, J. R., Bugeon, L. & Dallman, M. J. Mucosal inflammation at the respiratory interface: a zebrafish model. *Am J Physiol Lung Cell Mol Physiol* **310,** L551-61 (2016).

94. Zheng, W. *et al.* Comparative transcriptome analyses indicate molecular homology of zebrafish swimbladder and mammalian lung. *PLoS One* **6,** e24019 (2011).

95. Perrin, S., Rich, C. B., Morris, S. M., Stone, P. J. & Foster, J. A. The Zebrafish Swimbladder: A Simple Model for Lung Elastin Injury and Repair. *Connect. Tissue Res.* **40,** 105–112 (1999).

96. Robertson, G. N., McGee, C. A. S., Dumbarton, T. C., Croll, R. P. & Smith, F. M. Development of the swimbladder and its innervation in the zebrafish,Danio rerio. *J. Morphol.* **268,** 967–985 (2007).

97. Zhang, Y. *et al.* Manipulating the air-filled zebrafish swim bladder as a neutrophilic inflammation model for acute lung injury. *Cell Death Dis.* **7,** e2470 (2016).

98. Yin, A., Korzh, S., Winata, C. L., Korzh, V. & Gong, Z. Wnt Signaling Is Required for Early Development of Zebrafish Swimbladder. *PLoS One* **6,** e18431 (2011).

99. Winata, C. L. *et al.* Development of zebrafish swimbladder: The requirement of Hedgehog signaling in specification and organization of the three tissue layers. *Dev. Biol.* **331,** 222–236 (2009).

100. Massarsky, A., Prasad, G. L. & Di Giulio, R. T. Total particulate matter from cigarette smoke disrupts vascular development in zebrafish brain (Danio rerio). *Toxicol. Appl. Pharmacol.* **339,** 85–96 (2018).

101. Saad, M. *et al.* Xenobiotic metabolism in the zebrafish: a review of the spatiotemporal distribution, modulation and activity of Cytochrome P450 families 1 to 3. *J Toxicol Sci* **41,** 1–11 (2016).

102. Goldstone, J. V *et al.* Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. *BMC Genomics* **11,** 643 (2010).

103. Bräunig, J. *et al.* Time-dependent expression and activity of cytochrome P450 1s in early life-stages of the zebrafish (Danio rerio). *Environ. Sci. Pollut. Res.* **22,** 16319–16328 (2015).

104. Scornaienchi, M. L., Thornton, C., Willett, K. L. & Wilson, J. Y. Functional differences in the cytochrome P450 1 family enzymes from Zebrafish (Danio rerio) using heterologously expressed proteins. *Arch. Biochem. Biophys.* **502,** 17–22 (2010).

105. Kais, B., Schiwy, S., Hollert, H., Keiter, S. H. & Braunbeck, T. In vivo EROD assays with the zebrafish (Danio rerio) as rapid screening tools for the detection of dioxin-like activity. *Sci. Total Environ.* **590–591,** 269–280 (2017).

106. Ellis, L. D., Soo, E. C., Achenbach, J. C., Morash, M. G. & Soanes, K. H. Use of the zebrafish larvae as a model to study cigarette smoke condensate toxicity. *PLoS One* **9,** e115305 (2014).

107. Zhao, S. *et al.* Impact of maternal nicotine exposure on expression of myelin-related genes in zebrafish larvae. *Zebrafish* **11,** 10–16 (2014).

108. Parker, B. & Connaughton, V. P. Effects of Nicotine on Growth And Development in Larval Zebrafish. *Zebrafish* **4,** 59–68 (2007).

109. Massarsky, A. *et al.* Teratogenic, bioenergetic, and behavioral effects of exposure to total particulate matter on early development of zebrafish (Danio rerio) are not mimicked by nicotine. *Neurotoxicol Teratol* **51,** 77–88 (2015).

110. Palpant, N. J., Hofsteen, P., Pabon, L., Reinecke, H. & Murry, C. E. Cardiac development in zebrafish and human embryonic stem cells is inhibited by exposure to tobacco cigarettes and e-cigarettes. *PLoS One* **10,** e0126259 (2015).

111. Folkesson, M. *et al.* Differences in cardiovascular toxicities associated with cigarette smoking and snuff use revealed using novel zebrafish models. *Biol Open* (2016). doi:10.1242/bio.018812

112. Ho, D. H. & Burggren, W. W. Parental hypoxic exposure confers offspring hypoxia resistance in zebrafish (Danio rerio). *J Exp Biol* **215,** 4208–4216 (2012).

113. Baker, T. R., King-Heiden, T. C., Peterson, R. E. & Heideman, W. Dioxin induction of transgenerational inheritance of disease in zebrafish. *Mol Cell Endocrinol* **398,** 36–41 (2014).

114. Xu, N., Chua, A. K., Jiang, H., Liu, N. A. & Goodarzi, M. O. Early embryonic androgen exposure induces transgenerational epigenetic and metabolic changes. *Mol Endocrinol* **28,** 1329–1336 (2014).

115. Lombo, M. *et al.* Transgenerational inheritance of heart disorders caused by paternal bisphenol A exposure. *Env. Pollut* **206,** 667–678 (2015).

116. mouse human homoloy.

117. drosophila human homology.

118. D'Costa, A. & Shepherd, I. T. Zebrafish Development and Genetics: Introducing Undergraduates to Developmental Biology and Genetics in a Large Introductory Laboratory Class. *Zebrafish* **6,** 169–177 (2009).